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The Coinhibitory Receptor CTLA-4 Controls B Cell Responses by Modulating T Follicular Helper, T Follicular Regulatory, and T Regulatory Cells

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SUMMARY

The receptor CTLA-4 has been implicated in controlling B cell responses, but the mechanisms by which CTLA-4 regulates antibody production are not known. Here we showed deletion of CTLA-4 in adult mice increased Tfh and Tfr cell numbers and augmented B cell responses. In the effector phase, loss of CTLA-4 on Tfh cells resulted in heightened B cell responses, whereas loss of CTLA-4 on Tfr cells resulted in defective suppression of antigen-specific antibody responses. We also found that non-Tfr Treg cells could suppress B cell responses through CTLA-4 and that Treg and/or Tfr cells might downregulate B7-2 on B cells outside germinal centers as a means of suppression. Within the germinal center, however, Tfr cells potently suppress B cells through CTLA-4, but with a mechanism independent of altering B7-1 or B7-2. Thus, we identify multifaceted regulatory roles for CTLA-4 in Tfh, Tfr, and Treg cells, which together control humoral immunity.

INTRODUCTION

T follicular helper (Tfh) cells are a specialized subset of $CD4^+$ T cells that stimulate germinal center (GC) B cells to produce high-affinity antibodies. The critical role for Tfh cells in B cell responses is highlighted by the lack of class-switched antibodies in mice lacking Tfh cells (Crotty, 2011). Tfh cells are identified by expression of CXCR5, the chemokine receptor that directs them to GCs (Breitfeld et al., 2000; Crotty, 2011). Tfh cells also express high amounts of the transcription factor Bcl6, which is thought to control the Tfh cell program (Johnston et al., 2009; Yu et al., 2009; Nurieva et al., 2009). Tfh cells are controlled by positive costimulatory signals through the inducible T cell costimulator (ICOS) and CD28 receptors, as well as coinhibitory signals through programmed death 1 (PD-1). ICOS promotes Tfh cell generation and maintenance, whereas PD-1 inhibits Tfh differentiation and/or exit into the blood (Akiba et al., 2005; Choi et al., 2011; Good-Jacobson et al., 2010; Hams et al., 2011; Kawamoto et al., 2012; Sage et al., 2013).

T follicular regulatory (Tfr) cells are a newly defined, specialized effector subset of T regulatory (Treg) cells that suppress B cell responses (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Like Tfh cells, Tfr cells express high levels of CXCR5, which directs them to GCs. The ability of Tfr cells to suppress B cell responses might be unique to Tfr cells because $CXCR5^-$ Treg cells are unable to strongly suppress some GC B cell responses (Chung et al., 2011; Sage et al., 2013; Wollenberg et al., 2011). However, the precise role of Tfr versus non-Tfr Treg cells in controlling B cell responses remains undetermined. Tfr cells are controlled by positive and negative costimulatory signals, and ICOS and CD28 promote Tfr cell development (Linterman et al., 2011; Sage et al., 2013), whereas PD-1 attenuates both Tfr cell generation and suppressive function (Sage et al., 2013). It has been proposed that within the GC, the relative proportions of Tfr to Tfh cells (as well as their functional capacity) control B cell responses and not absolute numbers of either cell type (Sage et al., 2013).

Although CTLA-4 has been implicated in controlling B cell responses, the mechanism by which CTLA-4 regulates antibody production remains unknown. CTLA-4 is a key mediator of Treg cell function and also controls conventional T cells. CTLA-4 is constitutively expressed in Treg cell subsets, but induced upon activation in T conventional cells (Walker, 2013). Germline deletion of CTLA-4 results in fatal multiorgan inflammation within 2 to 4 weeks of age (Tivol et al., 1995; Waterhouse et al., 1995), as well as increased antibody levels (Bour-Jordan et al., 2003; Walker et al., 2003). Treg-specific deletion of CTLA-4 recapitulates this great increase in antibody production, pointing to an essential role for CTLA-4 on Treg cells in limiting B cell responses (Wing et al., 2008). However, it is not yet clear whether CTLA-4 suppresses B cell responses by controlling Tfr, Treg, and/or Tfh cells, due to the lethality associated with CTLA-4 global and Treg cell-specific deficiency, and the inability for blocking antibodies to target specific cells.

There are data supporting cell-intrinsic and cell-extrinsic mechanisms by which CTLA-4 exerts its effects (Corse and Allison, 2012; Walker and Sansom, 2011; Walunas et al., 1996; Wang et al., 2012). CTLA-4 binds to B7-1 (CD80) and B7-2 (CD86) with higher affinity than CD28. In vitro studies have demonstrated that CTLA-4 can attenuate B7-1 or B7-2 expression on dendritic cells either by downregulation or transendocytosis (Onishi et al., 2008; Qureshi et al., 2011; Wing et al., 2008). Whether CTLA-4 attenuates B7-1or B7-2 expression in vivo or

whether these CTLA-4 mediated cell-extrinsic mechanisms control B cell responses are still unclear.

Here we investigate cellular mechanisms by which CTLA-4 regulates B cell responses using CTLA-4 inducible knockout strategies. We analyzed how CTLA-4 controls Tfh, Tfr, Treg, and B cell responses. Our studies showed that CTLA-4 inhibited Tfh and Tfr cell differentiation and/or expansion. We also demonstrated that CTLA-4 mediated the suppressive capacity of differentiated Tfr cells. We did not find evidence that Tfr cells downregulate B cell B7-1 or B7-2 through CTLA-4 in GCs; however, Treg and/or Tfr cells might downregulate B7-2 on B cells outside the germinal center via CTLA-4. Our studies reveal multifaceted roles for CTLA-4 on Tfh, Tfr, and Treg cells in regulating humoral immunity.

RESULTS

T Follicular Regulatory Cells Express Large Amounts of CTLA-4

To begin to understand the function of CTLA-4 in controlling B cells, we assessed CTLA-4 expression in Tfh and Tfr cells. We immunized mice with NP-OVA (emulsified in CFA) subcutaneously (s.c.) and analyzed intracellular CTLA-4 expression in Tfh and Tfr in the draining lymph node (dLN) 7 days later. We identified Tfr cells as $CD4^+CXCR5^+ICOS^+FoxP3^+CD19^-$ cells and Tfh cells as $CD4^+CXCR5^+ICOS^+FoxP3^-CD19^-$ cells (Figure 1A). Tfr cells had extremely high expression of CTLA-4, whereas Tfh cells expressed more modest levels of CTLA-4 (Figure 1B). About 20% of Tfh and $ICOS^+$ ($CD4^+ICOS^+CXCR5^-FoxP3^-$) cells expressed CTLA-4, whereas $CD4^+ICOS^-CXCR5^-$ $FoxP3^-$ (DN) cells, a gate comprising mostly naive cells, had virtually no CTLA-4 expression (Figure 1C). About 80% of Tfr cells expressed CTLA-4, which was similar to $ICOS^+CXCR5^-$ Treg cells ($CD4^+ICOS^+CXCR5^-FoxP3^+$).

Because $ICOS^+CXCR5^-$ Treg cells highly express CTLA-4, we next determined whether ICOS and CTLA-4 expression were similarly high on Tfr cells. Costaining of dLN Tfr cells revealed that high ICOS expression correlated with high CTLA-4 expression (Figure 1D). Because increased IRF4 expression correlates with suppressive function in Tfr cells (Sage et al., 2013), we also costained Tfr cells for CTLA-4 and IRF4. IRF4 expression also correlated with increased CTLA-4 expression (Figure 1E). We next asked whether CTLA-4 is a marker for proliferating Tfr cells by using Ki67 to identify actively cycling cells. There was only a small increase in CTLA-4 expression on $Ki67^+$ cells compared to $Ki67^-$ Tfr cells (Figure 1F). In addition, we investigated whether high CTLA-4 expression correlated with PD-1 expression. CTLA-4 expression was only slightly higher on cells highly expressing PD-1 compared to cells with intermediate levels of PD-1 (Figure 1G). Together, these data indicate that CTLA-4 expression in Tfr cells correlates with ICOS and IRF4 and to a lesser extent with Ki67 and PD-1 expression.

We also compared CTLA-4 expression on Tfr cells from different anatomical locations, because these cells are not only found in the draining LN and spleen, but also in the circulation, Peyer's patches (PP), and skin (Kawamoto et al., 2014; Sage et al., 2013, 2014; Tsuji et al., 2009). Although not completely understood, Tfh (and Tfr) cells in the circulation have been postulated to represent distinct subsets and might represent memory cells (Craft, 2012; He et al., 2013; Locci et al., 2013; Sage et al., 2014; Sage et al., 2013). Most Tfr cells were positive for CTLA-4 regardless of their anatomical location (Figures 1H and 1I). However, Tfr cells from different anatomical locations had distinct surface-expression levels of ICOS (Figure 1J). These data indicate that CTLA-4 expression in Tfr cells is universal and expression correlates with functionally competent Tfr cells.

Inducible Global Deletion of CTLA-4 Results in Increased Tfr Differentiation and Increased Germinal Center B Cells

To assess the role of CTLA-4 in regulating B cell responses, we developed inducible deletion strategies. We crossed *Ctla4* floxed mice with UBC-ERT2-Cre (referred to as UBC-iCre) transgenic mice so that CTLA-4 could be deleted on all cells after the administration of tamoxifen (A.M.P. and A.H.S., unpublished data). We gave tamoxifen to UBC-$iCre^+Ctla4^{F/F}$ or UBC-$iCre^-Ctla4^{F/F}$ mice 3 days before immunization with NP-OVA (Figure 2A) to optimally and specifically delete CTLA-4 at the time of immunization. CTLA-4 was deleted on most Tfr and Tfh cells (Figure 2B; see also Figures S1A and S1B available online). Importantly, unlike germline deletion of CTLA-4, deletion of CTLA-4 in adult UBC-Cre mice did result in spontaneous inflammation during the immunization timeframe (data not shown). Thus, this strategy allowed us to delete CTLA-4 at the start of immunization, while circumventing potential indirect effects related to T cell development or autoimmunity.

We assessed how deletion of CTLA-4 affected the differentiation and maintenance of Tfh and Tfr cells. Tfh cells in CTLA-4 deleted mice were increased 2-fold compared to controls at day 9 after immunization (Figure 2C). ICOS expression was increased substantially on Tfh cells after CTLA-4 deletion (Figure 2D). Tfr cells were expanded ~5 fold after deletion of CTLA-4, whether expressed as a percentage of total $FoxP3^+$ cells (i.e., the precursors for Tfr cells) or of total $CD4^+$ T cells on day 9 postimmunization (Figures 2E and 2F). The total number of Tfr cells was 5-fold greater when CTLA-4 was deleted (Figure S1D). This increase in Tfr cells was also apparent when other gating strategies were used to identify Tfr cells (Figure S1C). Similar to Tfh cells, ICOS expression was also highly upregulated on Tfr cells after deletion of CTLA-4 compared to controls (Figure 2G).

To determine the relative proportion of Tfr cells compared to Tfh cells, we calculated the percentage of Tfr cells in the total $CXCR5^+CD4^+$ T cell population. The relative abundance of Tfr cells compared to Tfh cells was much higher after deletion of CTLA-4 compared to control mice in both the dLN and blood (Figure 2H). Therefore, deletion of CTLA-4 during the initiation of B cell responses results in increased Tfh and Tfr cells, with relatively greater increases in Tfr cells, altering the balance of T cells in the GC toward suppressive Tfr cells.

We next investigated the effect of this marked increase in Tfr cells on B cell responses in immunized mice in which CTLA-4 was deleted. We analyzed $CD19^+GL7^+FAS^+$ GC B cells from the dLN of mice after immunization as in Figure 2A and found increased percentages of GC B cells in the dLN after CTLA-4 deletion (Figure 2I). $CD138^+$ plasma cells, however, were not substantially increased (Figure S1E). Downregulation, or transendocytosis, of B7-1 and B7-2 has been proposed to

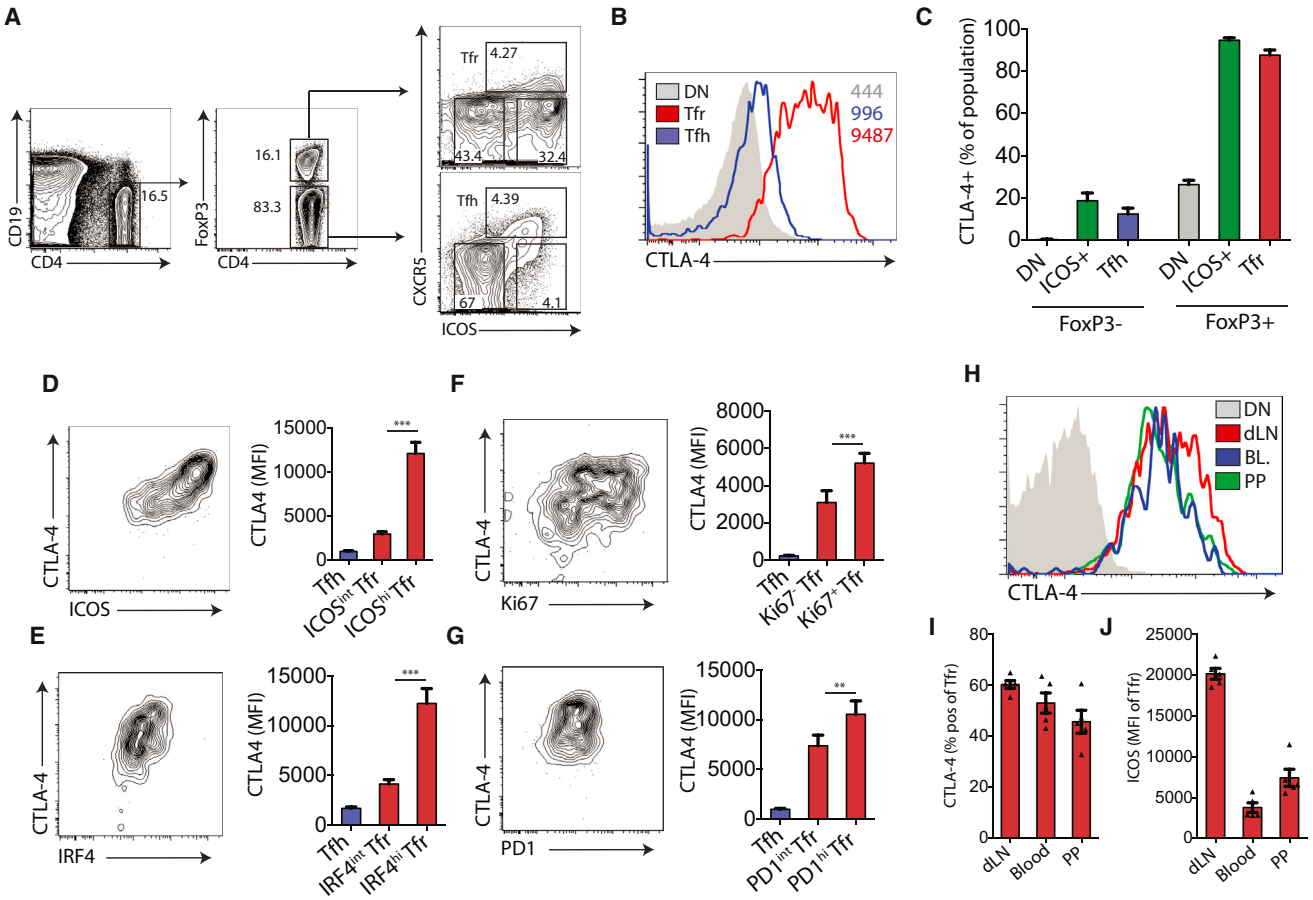

Figure 1. T Follicular Regulatory Cells Express High Amounts of CTLA-4

(A) Gating strategy to identify $CD4^+ICOS^+CXCR5^+FoxP3^-CD19^-$Tfh and $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$ Tfr cells from dLN of immunized mice.

(B) Histograms demonstrating intracellular CTLA-4 expression in Tfh and Tfr cells gated as in (A). DN represents $CD4^+ICOS^-CXCR^-FoxP3^-$ cells and correspond to naive $CD4^+$ T cells. Inset numbers indicate mean fluorescence intensity (MFI).

(C) Quantification of intracellular CTLA-4 expression in $FoxP3^-$ and $FoxP3^+$ cellular subsets. DN represents $CD4^+ICOS^-CXCR5^-$, $ICOS^+$ represents $CD4^+ICOS^+CXCR5^-$.

(D) CTLA-4 expression correlates with ICOS expression. Staining of ICOS and CTLA-4 in Tfr cells (left). Quantification of CTLA-4 in Tfr cells with intermediate (int) or high (hi) ICOS expression (right).

(E) CTLA-4 expression correlates with IRF4 expression. Staining of IRF4 and CTLA-4 in Tfr cells (left). Quantification of CTLA-4 in Tfr cells with intermediate (int) or high (hi) IRF4 expression (right).

(F) CTLA-4 and Ki67 expression. Staining of Ki67 and CTLA-4 in Tfr cells (left). Quantification of CTLA-4 in Tfr cells positive (+) or negative (–) for Ki67 expression (right).

(G) CTLA-4 and PD-1 expression. Staining of PD-1 and CTLA-4 in Tfr cells (left). Quantification of CTLA-4 in Tfr cells with intermediate (int) or high (hi) PD-1 expression (right).

(H and I) CTLA-4 expression in Tfr cells from dLN, blood (BL), or PP. DN represents $CD4^+ICOS^-CXCR5^-FoxP3^-$ cells. Quantification is shown (I).

(J) ICOS expression on Tfr cells from dLN, blood, and PP in as in (H).

All error bars contain SE.

be one mechanism by which CTLA-4 can control immune responses (Qureshi et al., 2011). We found no substantial changes in B7-1 or B7-2 expression on GC B cells in immunized CTLA-4-deleted mice compared to controls (Figure 2J). However, expression of B7-2 (but not B7-1) was significantly increased on total B cells in CTLA-4-deleted mice compared to controls.

To determine whether the increase in GC B cells resulted in increased antibody levels, we measured total immunoglobulin G1 (IgG1), total IgE, and NP-specific IgG1. Total IgG1 was higher in CTLA-4-deleted mice at d21. However, surprisingly, we did not find increases in antigen-specific antibody (Figure 2K). Interestingly, total IgE was much higher at d14 and d21 after immunization. To determine whether there were any spontaneous changes in serum Ig levels in unimmunized UBC-$iCre^+Ctla4^{F/F}$ mice at a later time point after deletion, we also assessed serum antibody levels in unimmunized UBC-$iCre^+Ctla4^{F/F}$ mice ~240 days after deletion. We found increases in IgG1 and IgE serum levels in these unimmunized CTLA-4-deleted mice (Figure 2L). Together, these data indicate that selective deletion of CTLA-4 at the time of initiation of a humoral immune response results in increased Tfh cells and an even greater increase in Tfr

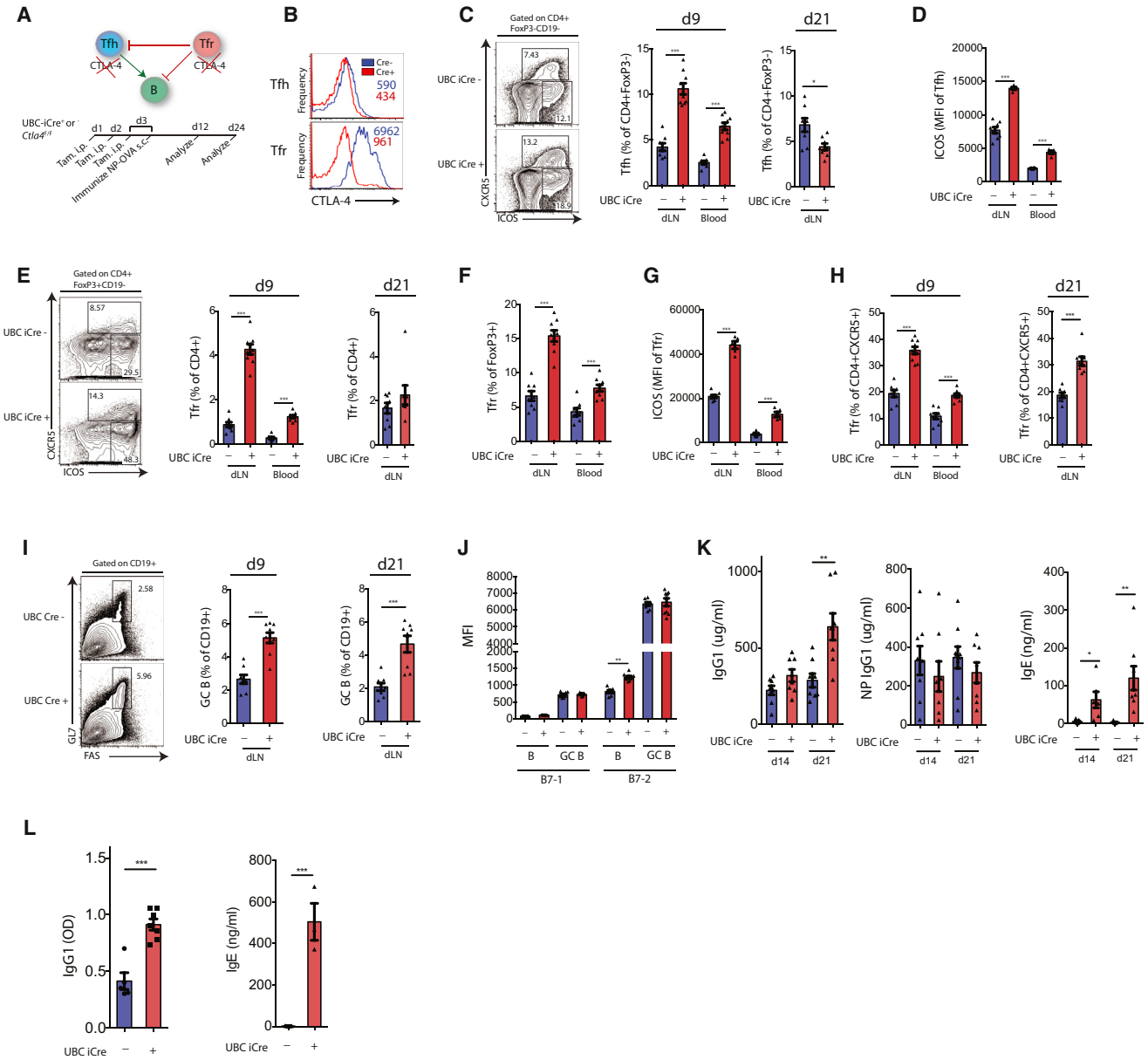

Figure 2. Inducible Global Deletion of CTLA-4 Results in Increased Tfr Cell Differentiation and Increased Germinal Center B Cells

(A) Schematic diagram of inducible deletion strategy to study the role of CTLA-4 in regulating B cell responses. UBC-iCre^{-}*Ctla4*$^{F/F}$ or UBC-iCre^{+}*Ctla4*$^{F/F}$ mice were given tamoxifen daily for 3 days and on the third day, immunized with NP-OVA s.c.; 9 or 21 days later, dLN or blood was harvested for analysis.

(B) Histogram demonstrating deletion of CTLA-4 in Tfh and Tfr cells. Tfh and Tfr cells were gated as in Figure 1A. Inset numbers indicate MFI.

(C) Tfh cell numbers are increased after total deletion of CTLA-4. Representative histograms of Tfh cell gating (left, at d9) and quantification of Tfh cells (right, at d9 and d21).

(D) Increased ICOS expression on Tfh cells after CTLA-4 deletion. ICOS expression on Tfh cells gated as in (C) at d9 postimmunization.

(E) Increased Tfr cell percentages after deletion of CTLA-4. Representative histograms of Tfr cell gating (left, at d9) and quantification of Tfr cells (right, at d9 and d21 postimmunization). Tfr cell percentages reported as a percentage of all CD4^{+} T cells.

(F) Tfr cell percentages reported as a percentage of all FoxP3^{+} T cells 9 days after immunization.

(G) ICOS expression on Tfr cells 9 days after immunization.

(H) Tfr cell percentage of total CD4^{+}CXCR5^{+} T cells 9 or 21 days after immunization.

(I) Enhanced germinal center B cells after CTLA-4 deletion. Gating of CD19^{+}GL7^{+}FAS^{+} germinal center (GC) B cells (left, at d9). Quantification of GC B cells (middle at d9, and right at d21 after immunization).

(J) Quantification of B7-1 and B7-2 on GC and total B cells from (I) 9 days after immunization.

(K) Quantification of serum antibody levels 14 and 21 days after immunization for total IgG1 (left), NP specific IgG1 (middle), or IgE (right).

(L) Quantification of serum antibody levels 240 days after CTLA-4 deletion in unimmunized mice. See also Figure S1.

All error bars contain SE.

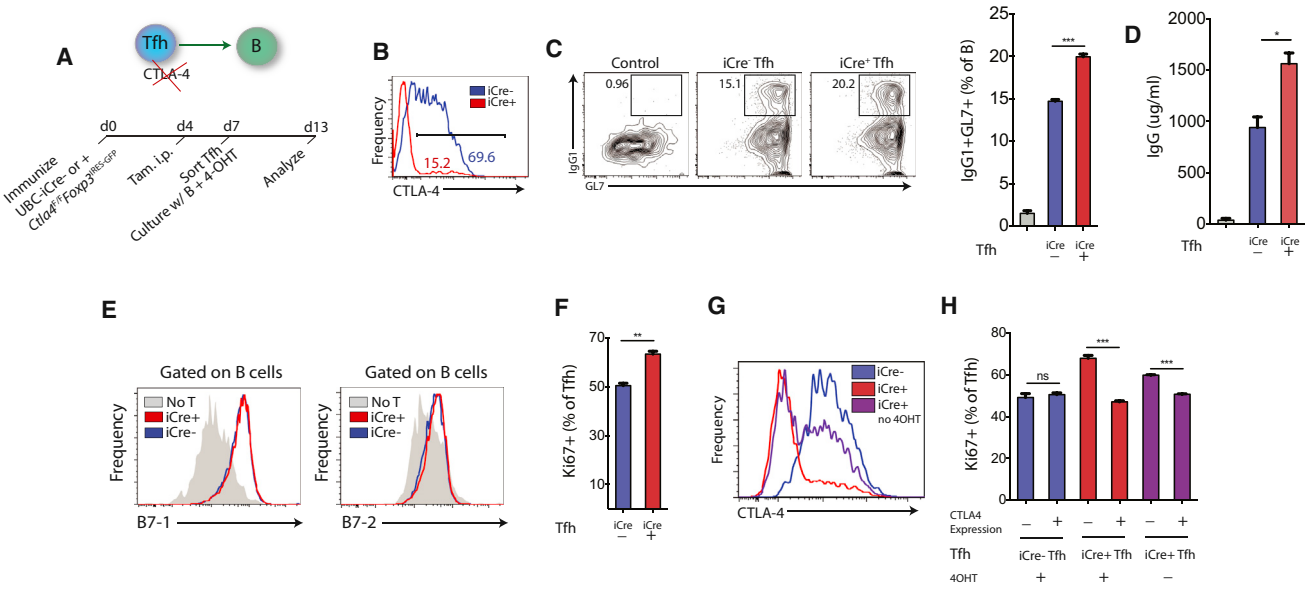

Figure 3. CTLA-4 Inhibits Tfh Stimulation of B Cells

(A) Schematic diagram of experimental approach. 10–20 UBC-iCre$^-$ or UBC-iCre$^+$ *Ctla4*$^{F/F}$ *Foxp3*$^{IRES\text{-}GFP}$ mice were immunized with NP-OVA s.c., and 4 days later tamoxifen was administered i.p.; 3 days later CD4$^+$ICOS$^+$CXCR5$^+$FoxP3$^-$CD19$^-$ (Tfh) cells were sorted and plated with CD19$^+$ cells (from dLN of immunized iCre$^-$ control mice) along with anti-CD3, anti-IgM, and 4OHT for 6 days.

(B) Histogram demonstrating expression of CTLA-4 on Tfh cells in stimulation assay after deletion. Numbers indicate percent positive based on gate.

(C) Enhanced stimulatory capacity of Tfh cells after deletion of CTLA-4. B cells from cocultures as in (A) were stained for GL7 and intracellularly for IgG1 to identify class-switched B cells. Gating strategy (left) and quantification (right).

(D) Enhanced stimulatory capacity of Tfh cells after deletion of CTLA-4 using NP-OVA. Cocultures were performed as in (A) but NP-OVA was added to wells instead of anti-CD3/IgM. 6 days later supernatants were analyzed for IgG.

(E) Expression of B7-1 (left) and B7-2 (right) on B cells from stimulation assays as in (A).

(F) Intracellular expression of Ki67 in Tfh cells from stimulation assays as in (A)

(G) CTLA-4 expression in Tfh cells from mice defined in (A) and also with Cre$^+$ mice injected with tamoxifen in vivo but not cultured with 4OHT to achieve the 50% Tfh deletion of CTLA-4.

(H) CTLA-4 inhibits Tfh stimulatory function in a cell intrinsic manner. Expression of Ki67 in CTLA-4 nonexpressing (–) or CTLA-4 expressing (+) Tfh cells from stimulation assays. See also Figure S2.

cells, as well as enhanced GC B cells and increased serum antibody levels.

Deletion of CTLA-4 in Tfh Cells Results in Enhanced Stimulatory Capacity

Next we assessed whether selective deletion of CTLA-4 on Tfh cells during the effector phase alters their stimulatory capacity. To do this, we utilized an in vitro B cell stimulation assay. We immunized UBC-iCre$^+$*Ctla4*$^{F/F}$*Foxp3*$^{IRES\text{-}GFP}$ or UBC-iCre$^-$ *Ctla4*$^{F/F}$*Foxp3*$^{IRES\text{-}GFP}$ mice and sorted Tfh (CD4$^+$ICOS$^+$CXCR5$^+$FoxP3$^-$CD19$^-$) cells from dLNs. The sorted Tfh cells were cultured with wild-type (WT) B cells (from dLN of NP-OVA immunized mice) in the presence of anti-CD3, anti-IgM, and 4-hydroxy-tamoxifen (4OHT) for 6 days (Figure 3A). After culture, control iCre$^-$ Tfh cells had substantial CTLA-4 expression, but use of tamoxifen in vivo combined with culture in 4OHT resulted in substantial deletion of CTLA-4 in iCre$^+$ Tfh cells (Figure 3B).

To compare the capacity of CTLA-4 deleted and expressing Tfh cells to stimulate B cell class-switch recombination, we analyzed B cells from cultures. CTLA-4 deleted Tfh cells stimulated B cells to undergo class-switch recombination to IgG1 to a greater extent than CTLA-4 expressing (iCre$^-$) Tfh cells (Figure 3C). This increase in class-switch recombination was accompanied by increases in IgG in culture supernatant when NP-OVA was used to stimulate cells (Figure 3D). Despite increases in antibody, there were comparable numbers of Tfh cells, resulting from slight increases in cell death offsetting increased proliferation (Figure S2A). B cells were comparable in number and were phenotypically similar (Figures S2A–S2D). B cells from anti-CD3 and anti-IgM stimulation assays had similar upregulation of B7-1 and B7-2 expression after culture with CTLA-4-expressing or CTLA-4-deleted Tfh cells (Figure 3E). Because we have found that Ki67 expression in Tfh cells functionally predicts their stimulatory capacity (Sage et al., 2014), we analyzed Ki67 expression in Tfh cells after coculture with B cells. Ki67 was increased in CTLA-4 deleted Tfh cells compared to CTLA-4 expressing controls (Figure 3F).

To determine whether this increase in Ki67 is a cell-intrinsic or cell-extrinsic effect of CTLA-4 deletion in these stimulation assays, we cultured B cells with CTLA-4 control (iCre$^-$ cultured with 4OHT), CTLA-4 deleted (iCre$^+$ cultured with 4OHT) or CTLA-4 50% deleted from Tfh (iCre$^+$ mice injected with tamoxifen in vivo but not cultured with 4OHT to achieve the 50% Tfh deletion of CTLA-4). This strategy enabled us to compare a range of CTLA-4 deleted Tfh cells (Figure 3G). We compared Ki67 expression in the CTLA-4 expressing and CTLA-4

nonexpressing populations and found that Tfh cells with deleted CTLA-4 had a higher percentage of Ki67-positive cells compared to Cre^- controls, but that CTLA-4 expressing cells in the same well as CTLA-4 deleted cells did not have an increased percentage of Ki67-positive cells (Figure 3H). These experiments show that CTLA-4 has a cell-intrinsic function in Tfh cells to suppress their stimulatory function.

Inducible Deletion of CTLA-4 in Treg Cells Results in Increased Tfr Cells and Increased Germinal Center B Cells In Vivo

We next investigated the role of CTLA-4 on Tfr cells. We bred *Ctla4* floxed mice to *Foxp3*-ERT2-Cre-GFP knockin (Rubtsov et al., 2010) (referred to as $Foxp3^{iCre/iCre}$) mice to generate a mouse strain that conditionally deletes CTLA-4 only on $FoxP3^+$ Treg cell subsets only after administration of tamoxifen. This strategy allows for deletion of CTLA-4 on Tfr cells (and other $FoxP3^+$ Treg subsets) at the time of immunization, circumventing lethal spontaneous inflammation associated with germline deletion of CTLA-4 on $FoxP3^+$ Treg cells. We gave tamoxifen to $Foxp3^{iCre/iCre}Ctla4^{F/F}$ (referred to as F/F) or $Foxp3^{iCre/iCre}Ctla4^{F/+}$ (referred to as F/+) mice daily for 3 days and immunized with NP-OVA (Figure 4A). Tamoxifen deleted CTLA-4 in Tfr cells from F/F mice but not F/+ mice (Figures 4B and 4C and Figures S3A–S3C). Notably, $Foxp3^{iCre/iCre}$ mice have attenuated percentages of Tfr cells expressing CTLA-4 and lower T cell CXCR5 expression compared to non-Cre expressing mice, a phenomenon that is due to altered FoxP3 expression related to the $Foxp3^{iCre}$ knockin allele and not due to CTLA-4 floxed alleles (Figure S3B). We controlled for this alteration by comparing $Foxp3^{iCre/iCre}Ctla4^{F/F}$ and $Foxp3^{iCre/iCre}Ctla4^{F/+}$ mice. When we analyzed Tfr development in these strains, we found substantial increases in Tfr cells in CTLA-4 Treg-deleted mice compared to controls (Figure 4D and Figures S3D and S3E). The increase in Tfr cells in CTLA-4 Treg-deleted mice was due, in part, to substantial increases in total Treg cells (Figure 4E). Additionally, Tfr ICOS expression was greatly enhanced upon deletion of CTLA-4 (Figure 4F). The substantial increase in Tfr cells upon deletion of CTLA-4 was also associated with a very modest decrease in cell death (Figure 4G). Therefore, deletion of CTLA-4 in Treg cells results in enhanced Tfr differentiation and/or maintenance associated with increased ICOS expression.

Next we determined whether deletion of CTLA-4 on Treg cells changed the differentiation and/or phenotype of Tfh cells. We found significant increases in the percentage of Tfh cells in immunized mice that had CTLA-4 deleted on Treg cells (Figure 4H). Unlike total deletion of CTLA-4, however, Tfh cells in the dLN did not have a significant increase in ICOS expression as a result of deletion of CTLA-4 on Treg cells (Figure 4I). When we compared the percentage of Tfr cells in all $CXCR5^+$ $CD4^+$ T cells (and therefore the percentage of Tfr cells of all CD4 effectors in the GC), we found that Tfr cells comprised a much higher proportion of the $CXCR5^+$ $CD4^+$ T cell population in CTLA-4 Treg-deleted mice, compared to control mice (Figure 4J).

We next investigated GC B cells in the immunized CTLA-4 Treg-deleted mice to determine whether deletion of CTLA-4 on Treg cell subsets can recapitulate the enhanced GC B cells observed in mice in which CTLA-4 is inducibly deleted globally (UBC-Cre). We analyzed $GL7^+FAS^+$ GC B cells and found that deletion of CTLA-4 on Tfr and other Treg cell subsets resulted in increased GC B cell percentages and total numbers compared to controls (Figure 5A and Figure S4A). The increased GC B cells in CTLA-4 Treg-deleted mice had almost identical expression of B7-1 and B7-2 compared to nondeleted control mice (Figure 5B-C). However, total B cells had slightly higher expression of B7-2 (but not B7-1) after deletion of CTLA-4 on Treg cells, similar to global inducible CTLA-4 deletion. Despite increases in GC B cells, total serum IgG1 was not significantly altered in CTLA-4 deleted mice (Figure 5D). The increase in GC B cells was not be recapitulated in other Cre strains such as CD19 Cre (which deletes CTLA-4 on B cells) (Figure S4B). Together, these data indicate that selective deletion of CTLA-4 on Treg cell subsets at the beginning of antigen challenge results in increased Tfh cells and Tfr cells (with a marked increase in Tfr to Tfh ratio) and increased GC B cells.

Inducible Deletion of CTLA-4 on Tfr Cells after Differentiation Results in Decreased Suppressive Function In Vitro and In Vivo

Although GC B cells were increased in both the global and Treg-specific CTLA-4 deletion models, we did not find significant changes in antigen-specific antibody responses (although we did measure increases in total IgE and IgG1 after global deletion). We hypothesized that increases in Tfr cell numbers upon deletion of CTLA-4 might compensate for defective suppressive capacity of Tfr cells. Therefore, we further examined the role of CTLA-4 specifically on Tfr cells in regulating Tfr suppressive function. First, we assessed the effect of CTLA-4 on Tfr cell suppression utilizing in vitro suppression assays. We immunized UBC-$iCre^+Ctla4^{F/F}Foxp3^{IRES\text{-}GFP}$ ($iCre^+$) or UBC-$iCre^-Ctla4^{F/F}$ $Foxp3^{IRES\text{-}GFP}$ (WT) control mice with NP-OVA, gave tamoxifen on d4 (to start the deletion process so CTLA-4 can be efficiently deleted at the start of the in vitro assay) and sorted Tfr (sorted as $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$) cells on d7. The Tfr cells were cultured with B cells and Tfh cells (both sorted from immunized UBC-$iCre^-$ $Ctla4^{F/F}Foxp3^{IRES\text{-}GFP}$ WT mice) in the presence of anti-IgM, anti-CD3, and 4-OHT (Figure 6A-B). Importantly, precultured cells did not have any change in differentiation or surface phenotype because CTLA-4 had not been fully deleted (Figure S5A).

When we analyzed B cells from suppression assays, we found that WT Tfr cells almost completely suppressed class-switch recombination to IgG1, whereas $iCre^+$ Tfr cells suppressed class-switch recombination to IgG1 slightly less well (Figures 6C and 6D). Conventional Treg cells (sorted as $CD4^+FoxP3^+$ $ICOS^-CXCR5^-$) were able to attenuate class-switch recombination, but to a much lesser degree compared to Tfr cells (Figure 6D). Similar to Tfr cells, suppression by conventional Treg cells was attenuated with CTLA-4 deletion (Figure 6D). The difference in B cell responses after CTLA-4 deletion on Tfr cells was not due to altered Tfr numbers (Figure S5B). We have found that downregulation of the GC activation marker GL7 on B cells is more sensitive than class-switch recombination in determining Tfr suppressive capacity. The reduced suppressive capacity of CTLA-4 deleted Tfr cells was also evidenced by the increased percentage of $GL7^+$ B cells in cultures with CTLA-4 deleted Tfr cells (Figure 6E). Despite small changes in suppressive function,

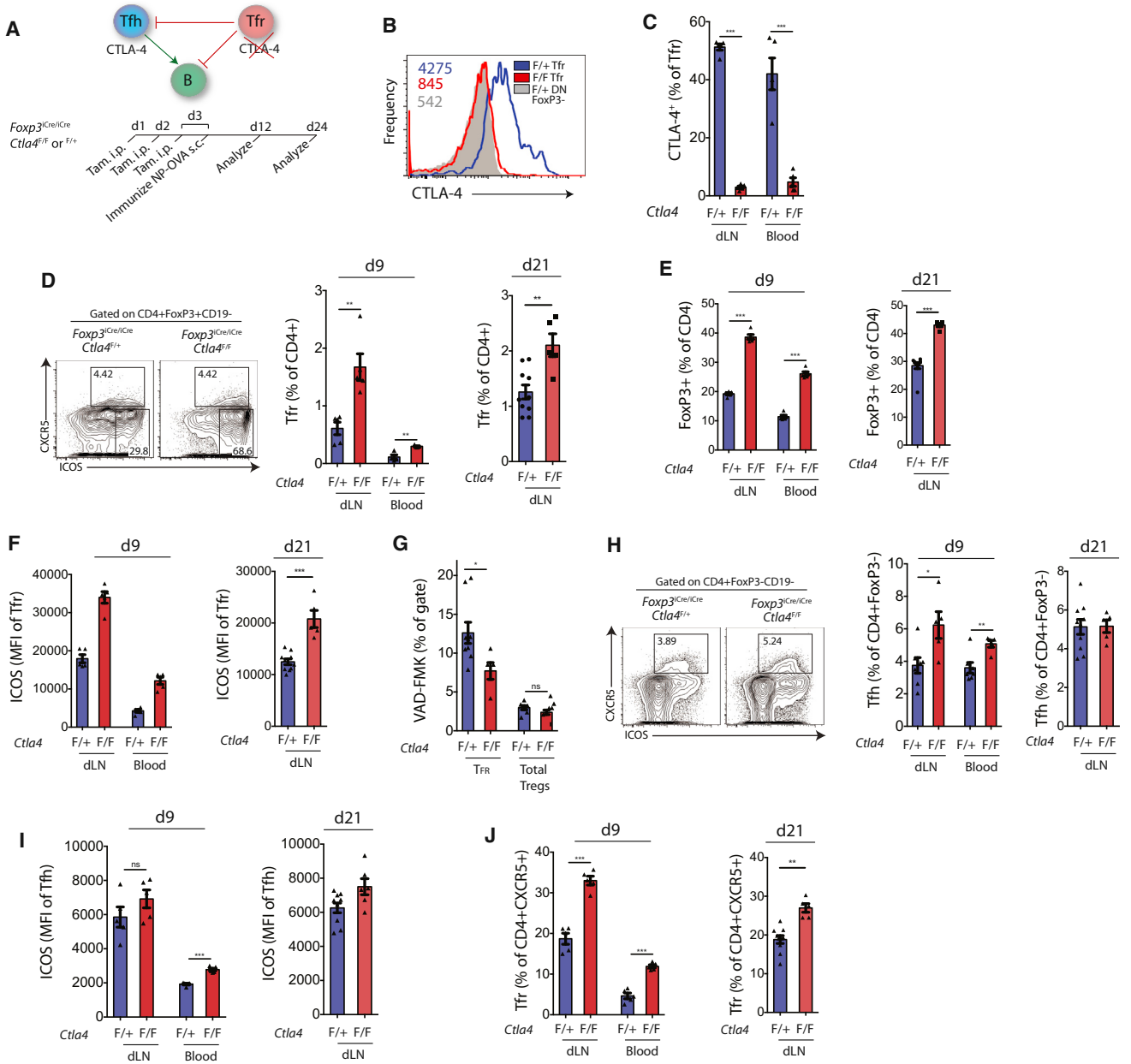

Figure 4. Inducible Deletion of CTLA-4 in Treg Cells Results in Increased Tfr and Tfh Cells

(A) Schematic diagram of experiment. *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/+}$ or *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/F}$ mice were injected with tamoxifen daily for 3 days before immunization with NP-OVA s.c. 9 or 21 days later dLN and blood were collected for analysis.

(B) Histogram showing deletion of CTLA-4 in Tfr cells. Cre^{-} ICOS^{-}CXCR5^{-}FoxP3^{-} cells (Cre- DN FoxP3^{-}) are included as controls. Inset numbers indicate MFI.

(C) Quantification of CTLA-4 staining in Tfr cells.

(D) Increased Tfr cell percentages after Treg cell-specific deletion of CTLA-4. Representative Tfr staining, gated on FoxP3^{+} cells (left, at d9), and Tfr quantification, as a percentage of total CD4^{+} T cells (right, at d9 and d21).

(E) Quantification of FoxP3^{+} cells after Treg cell-specific CTLA-4 deletion 9 and 21 days after immunization.

(F) Increased ICOS expression on Tfr cells after CTLA-4 deletion. ICOS expression was quantified on Tfr cells 9 and 21 days after immunization.

(G) Cell death of Tfr cells after deletion of CTLA-4 in vivo. Cell death in Tfr cells and total Treg cells was measured by staining with the activated caspase staining reagent VAD-FMK 9 days after immunization.

(H) Increased Tfh cell percentages after Treg cell-specific CTLA-4 deletion. Representative gating (left, at d9) and quantification (right, at d9 and d21) is shown.

(I) ICOS expression on Tfh cells after Treg-specific CTLA-4 deletion 9 or 21 days after immunization.

(J) Tfr cells are more abundant compared to Tfh cells in the germinal center. Percentage of Tfr cells of all CD4^{+}CXCR5^{+} cells is shown 9 or 21 days after immunization. See also Figure S3.

All error bars contain SE.

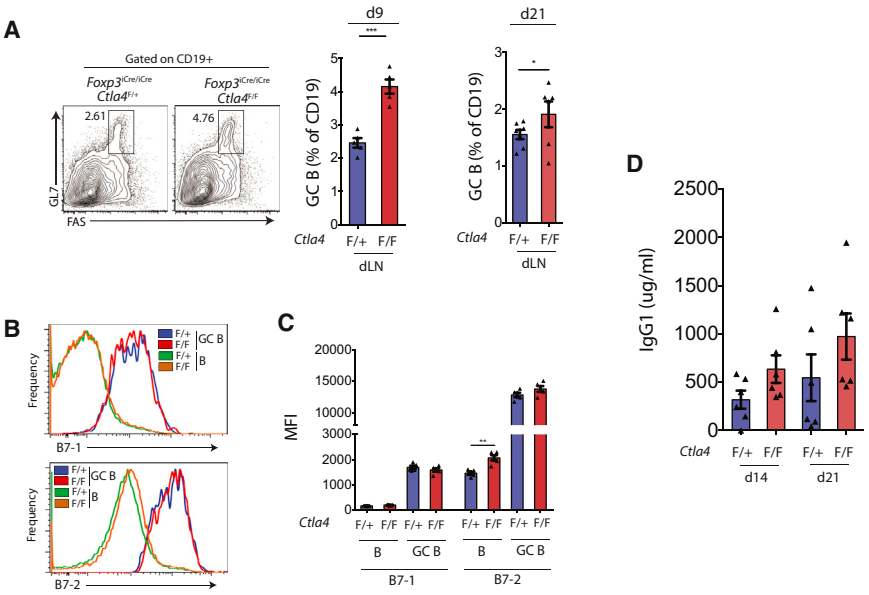

Figure 5. Inducible Deletion of CTLA-4 in Treg Cells Results in Increased Germinal Center B Cells In Vivo

(A) Enhanced GC B cell responses after Treg cell-specific deletion of CTLA-4. *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/+}$ or *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/F}$ mice were injected with tamoxifen daily for 3 days before immunization with NP-OVA s.c. 9 or 21 days later, dLN was collected for analysis.

(B and C) Expression of B7-1 and B7-2 on total CD19$^+$ B cells or on CD19$^+$GL7$^+$FAS$^+$ GC B cells 9 days after immunization. Representative histograms (B) and quantification (C) are shown.

(D) Serum IgG1 levels. Serum IgG1 was analyzed in mice 14 or 21 days after immunization as in (A). See also Figure S4.

All error bars contain SE.

the CTLA-4 deleted Tfr cells were able to suppress B7-1 expression levels on the surface of B cells similarly to WT Tfr cells (Figure 6F).

Activated Tfh cells cultured with B cells upregulate Ki67 and maintain levels of the transcription factor Bcl6 (Sage et al., 2014). When we assessed Bcl6 and Ki67 levels in Tfh cells in these suppression assays, we found that WT Tfr cells attenuated Tfh Bcl6$^+$Ki67$^+$ levels by ~70 percent, in marked contrast to CTLA-4 deleted Tfr cells, which were only able to attenuate Tfh Bcl6$^+$Ki67$^+$ levels by ~30 percent (Figure 6G). The difference in suppression was not due to altered B cell IDO, Tfr GITR, or Tfr IL-10 production (Figures S5C–S5E). Together, these data indicate that deletion of CTLA-4 during the effector phase diminishes Tfr suppressive capacity.

To determine whether deletion of CTLA-4 results in cell-intrinsic differences in the activation state of the Tfr cell, we developed a plate-bound assay to signal through CD28 and CTLA-4 in Tfr cells in the absence of other cell types. In this assay, we cultured sorted WT or iCre$^+$ Tfr cells on anti-CD3 and rB7-2-Fc coated plates. B7-2 was used, and not B7-1, to eliminate the possibility of B7-1 signaling into the Tfr cell through a B7-1:PD-L1 interaction (Butte et al., 2007). During culture, we eliminated CTLA-4 on Tfr cells with 4OHT and analyzed cultures 4 days later. We found that deletion of CTLA-4 on Tfr cells did not result in loss of FoxP3 expression but resulted in higher ICOS expression (Figures S5F and S5G).

Next we determined whether deletion of CTLA-4 only on Tfr cells and only during suppression altered B cell responses in vivo. To do this, we immunized *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/+}$ or *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/F}$ mice with NP-OVA s.c. and 6 days later gave tamoxifen to start deletion. Twenty-four hr later, we sorted total CD4$^+$ CXCR5$^+$ICOS$^+$CD19$^-$ cells (which contain Tfh and Tfr cells, but not other cellular subsets) and adoptively transferred these cells to *Cd28*$^{-/-}$ mice (which cannot generate Tfh and Tfr cells) that were then immunized with NP-OVA and given tamoxifen (Figure 6H). Because the transferred cells have an inducible Cre under the FoxP3 promoter, and only Tfh and Tfr cells were transferred, administration of tamoxifen to the recipients deletes CTLA-4 only on Tfr cells. When we analyzed recipients after adoptive transfer and deletion, we found that deletion of CTLA-4 on Tfr cells led to an overall increase in Tfh cells in recipients (Figure 6I), and this increase was not due to Tfr cell conversion into Tfh cells (Figure S5I). These results show that CTLA-4 deleted Tfr cells have reduced ability to suppress Tfh cells compared to control Tfr cells. Importantly, deletion of CTLA-4 on Tfr cells led to a substantial increase in NP-specific IgG, demonstrating diminished suppressive capacity of downstream B cell responses by Tfr cells after CTLA-4 deletion (Figure 6J). Although it is likely that this antibody originates from the GC responses, extrafollicular pathways might also contribute. The increased antibody produced in Tfr CTLA-4-deleted mice had a lower NP2/NP16 ratio, suggesting that the increased antibody might be of lower affinity (Figure S5H). Additionally, GC B cell numbers and plasma cell percentages were increased, although this did not reach statistical significance (Figure S5H). Unlike Tfr cells, conventional Treg cells were not able to suppress Tfh-mediated antigen-specific antibody production in *Cd28*$^{-/-}$ recipients (Figure S5J). Although we found substantial increases in antigen-specific antibody responses upon deletion of CTLA-4 in Tfr cells, B7-1 and B7-2 expression on GC and total B cells were not altered (Figures 6K and 6L). Together, these data indicate that CTLA-4-deleted Tfr cells exhibit defective B cell suppression using in vitro and in vivo assays of Tfr cell function.

Deletion of CTLA-4 in Tfr Cells Results in Enhanced B Cell Responses in Peyer's Patches

Because Tfr cells and Tfh cells are present in the germinal centers of PP (Kawamoto et al., 2014), we also investigated whether deletion of CTLA-4 in Tfr cells affects B cell responses in PP of the gut using both the UBC-iCre$^+$ CTLA-4$^{F/F}$ and *Foxp3*$^{iCre/iCre}$ CTLA-4$^{F/F}$ strains. We gave mice from each strain tamoxifen on 3 consecutive days, and analyzed PP for Tfh, Tfr, and B cell responses at several time points after tamoxifen administration. Global inducible deletion of CTLA-4 resulted in substantial deletion of CTLA-4 in both Tfh and Tfr cells of PP, similar to LN draining sites of immunization (Figures S6A and S6B). Total, but not

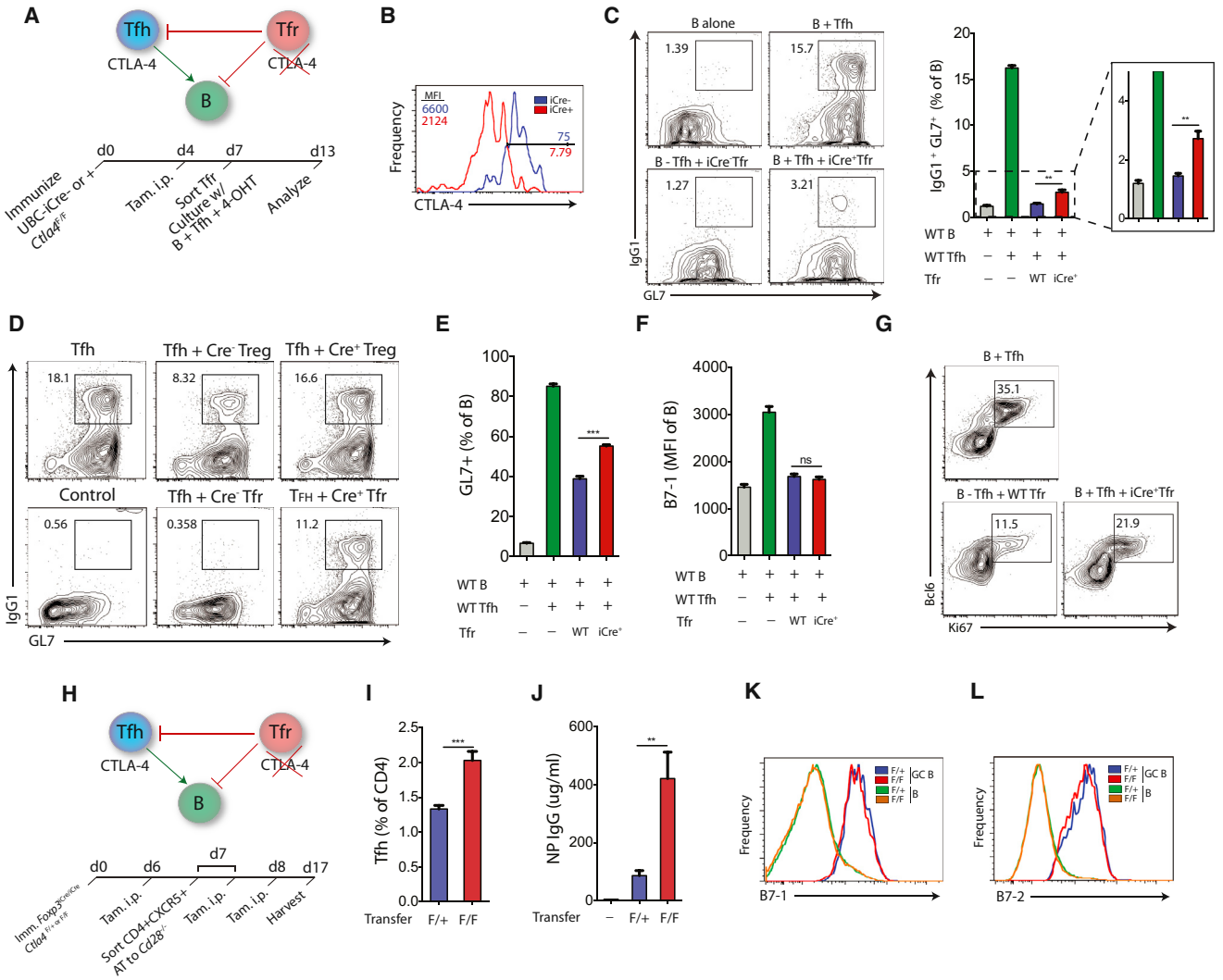

Figure 6. Inducible Deletion of CTLA-4 on Tfr Cells during Suppression Results in Decreased Suppressive Function In Vitro and In Vivo

(A) Schematic representation of experiment. Twenty UBC-iCre^{-}*Ctla4*$^{F/F}$*Foxp3*$^{IRES\text{-}GFP}$ (WT) or UBC-iCre^{+}*Ctla4*$^{F/F}$*Foxp3*$^{IRES\text{-}GFP}$ (iCre) mice were immunized with NP-OVA s.c. and 4 days later mice were given tamoxifen i.p.; 3 days later CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{-}CD19^{-} (Tfh) and CD19^{+} B cells from iCre- WT mice were cultured with anti-CD3, anti-IgM, and 4OHT. CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{+}CD19^{-} Tfr cells from WT or iCre^{+} mice were added. Cells were analyzed 6 days later.

(B) Deletion of CTLA-4 in Tfr cells after suppression assay as in (A). Tfr cells from cultures were identified as CD4^{+}FoxP3^{+}CD19^{-} cells.

(C) Deletion of CTLA-4 in Tfr cells results in decreased suppression of class-switch recombination. Representative plots of B cells showing GL7^{+}IgG1^{+} class-switched B cells from suppression assays detailed in (A; left). Quantification of class-switched B cells (right).

(D) Effect of deletion of CTLA-4 in conventional Treg cells in suppression assays. Experiments were performed as in (A) except CD4^{+}ICOS^{-}CXCR5^{-}FoxP3^{+} conventional Treg cells, or Tfr cells, were cultured with B cells. "Control" indicates B cells alone.

(E) GL7 expression on B cells from suppression assays as in (A).

(F) B7-1 expression on B cells from suppression assays as in (A).

(G) Deletion of CTLA-4 in Tfr cells results in decreased suppression of Tfh cells. Tfh cells from suppression assays in (A) were intracellularly stained for Ki67 and Bcl6. Tfh cells were identified as CD4^{+}FoxP3^{-}CD19^{-} cells from cultures. Representative plots are shown.

(H) Deletion of CTLA-4 in Tfr cells after differentiation results in diminished suppressive capacity in vivo. Schematic representation of assay. *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/F}$ or *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/+}$ mice were immunized and tamoxifen was administered 1 day before sorting total CD4^{+}ICOS^{+}CXCR5^{+}CD19^{-} (Tfh and Tfr) cells on day 6. Sorted cells were adoptively transferred to *Cd28*$^{-/-}$ mice that were immunized with NP-OVA and tamoxifen was administered on days 0 and 1 after immunization. Organs were harvested 9 days later for analysis.

(I) Quantification of Tfh cells in recipient mice from transfers in (H).

(J) NP-specific IgG serum levels from recipient mice in transfers as in (H).

(K) B7-1 and (L) B7-2 expression on total CD19^{+} (B) or CD19^{+}GL7^{+}FAS^{+} (GC B) B cells from transfers in (H). See also Figure S5.

All error bars contain SE.

Treg cell-specific, deletion of CTLA-4 resulted in slight increases in PP Tfh cell percentages (Figure 7A-B). In contrast, Tfr cells were enhanced as a percentage of $CD4^+$ (or of $FoxP3^+$) cells when CTLA-4 was deleted global or selectively in Treg cells (Figure 7C and Figure S6C), and proportions of Tfr cells were increased compared to Tfh cells using both approaches (Figure 7D). Therefore, in the absence of CTLA-4 on Treg cells, there are increased Tfr percentages in PP, similar to skin dLNs.

Next we investigated B cell responses in the PP from mice with global and Treg cell-specific deletion of CTLA-4. CTLA-4 deletion resulted in an increase in GC B cells, but not plasma cells, in PP (Figure 7E and Figure S6D). Deletion of CTLA-4 on all cells or Treg cells did not alter B7-1 or B7-2 expression on GC B cells (Figures 7F and 7G). However, as in skin dLNs, we did detect small but significant increases in expression levels of B7-2, but not B7-1, on total B cells after CTLA-4 deletion compared to nondeleted controls. Importantly, serum IgA levels were increased in mice after CTLA-4 deletion compared to nondeleted controls (Figure 7H). Together, these data indicate that deletion of CTLA-4 in Treg cells results in defective suppression, manifest as enhanced B cell responses in the PP. This phenotype mirrors the phenotype of skin dLN, suggesting that altered function of B cell responses in the PP in CTLA-4-deleted mice might be due to defective Tfr function.

DISCUSSION

Although CTLA-4 has been implicated in modulating B cell responses, the inability to selectively delete or inhibit CTLA-4 on defined cell types has led to confusion about the precise role(s) of CTLA-4 in controlling humoral immunity (Walker and Sansom, 2011). Here we utilize inducible CTLA-4 gene deleted mice to assess the role of CTLA-4 in B cell responses and identify functions for CTLA-4 on multiple T cell subsets in regulating humoral immunity. We find that CTLA-4 inhibited Tfh cell differentiation and/or maintenance and effector function. In addition, CTLA-4 potently inhibited Tfr cell differentiation and/or maintenance but was required in vivo for Tfr cells to fully suppress antigen-specific B cell responses in germinal centers. We also find evidence that non-Tfr Treg cells and/or Tfr cells might suppress B cell responses by CTLA-4-mediated downregulation of B7-2 presumably outside the germinal center. Therefore our work uncovers multiple roles for CTLA-4 on Tfh, Tfr, and Treg cells in regulating humoral immunity.

Global deletion of CTLA-4 at the time of immunization resulted in increased Tfh cells. Treg-specific deletion of CTLA-4 also resulted in increased Tfh cell differentiation but was not as substantial as global CTLA-4 deletion. Therefore, Tfh cells are regulated by Tfh-expressed CTLA-4, as well as Tfr-expressed CTLA-4. However, it is important to note that there might be differences in the deletion models, which alter Tfh percentages. For instance, in the Treg cell-specific CTLA-4 deletion model, control mice have only one functional allele of CTLA-4. Beyond differentiation, CTLA-4 also inhibits the B cell stimulatory function of Tfh cells, consistent with previous studies that demonstrate a specific role for CTLA-4 on T conventional cells, presumably separately from Treg cells (Greenwald et al., 2001; Ise et al., 2010; Walker, 2013; Walker et al., 2002). We did not detect any substantial changes in B7-1 or B7-2 levels on B cells from Tfh and B cell cocultures. Furthermore, cultures of B cells with CTLA-4 expressing plus CTLA-4 nonexpressing Tfh cells revealed cell intrinsic functional differences between these Tfh cell populations. Therefore, CTLA-4 likely functionally impedes Tfh cell differentiation and B cell stimulation by altering signaling into Tfh cells.

The machinery responsible for limiting Tfr cell differentiation and function are still relatively unknown. Previous studies have demonstrated roles for PD-1, Blimp-1, and Id2/Id3 in inhibiting Tfr differentiation (Linterman et al., 2011; Miyazaki et al., 2014; Sage et al., 2013). Here we show that CTLA-4 inhibits Tfr cell differentiation and/or maintenance. Deletion of CTLA-4 resulted in enhanced Tfr cell differentiation that was accompanied by substantially increased ICOS expression. Anti-CTLA-4 therapy similarly increases the frequency of $ICOS^+$ T cells in mouse models and human patients (Ng Tang et al., 2013).

In marked contrast to PD-1, CTLA-4 does not inhibit but promotes the suppressive capacity of Tfr cells. To assess the role of CTLA-4 on Tfr cells during suppression, we performed transfer and deletion studies to circumvent the effects of CTLA-4 on Tfr cell expansion and/or indirect effects from CTLA-4 on other cell types. In these experiments, we found clear enhancement of antigen-specific antibody responses when Tfr cells lacked CTLA-4. These findings contrast with global or Treg cell-specific deletion assays of CTLA-4 in which we did not measure increased antigen-specific antibody production but instead found increases in total IgG1 and IgE. We hypothesize that the increase in IgE and total IgG1 during global or Treg cell-specific CTLA-4 deletion might be due to defective Treg cell and/or Tfr suppression of B cells outside the germinal center (possibly via the extra follicular pathway of B cell activation near the T-B border). This suppression might be at least partially due to B7-2 downregulation or transcytosis, because we found that B7-2 expression was lower on total (but not GC) B cells upon CTLA-4 deletion in both global and Treg cell-specific CTLA-4 deletion models in both the dLN and PP. Although downregulation of B7-2 on total B cells was not observed in Tfr transfer and CTLA-4 deletion models, these transfer models of fully differentiated Tfr cells might limit the duration of Tfr cells outside the germinal center due to high CXCR5 expression associated with full differentiation at the time of transfer.

Although increased total IgG1 and IgE can be explained by lack of Treg cell (and/or Tfr) suppression through downregulation of B7-2, enhanced antigen-specific antibody would be expected in global or Treg cell-specific deletion models if CTLA-4 deleted Tfr cells were defective in their capacity to suppress GC B cells. However, we did not observe increased antigen-specific antibody responses in global or Treg cell-specific CTLA-4 deleted mice. We hypothesize that Tfr cells in global or Treg cell-specific CTLA-4 deleted mice are indeed defective in suppression but that the substantial increases in Tfr cell numbers during differentiation, combined with suppression mechanisms that are independent of CTLA-4, override these defects. We hypothesize that there might be multiple suppression mechanisms built into the Tfr program as a means to prevent autoimmunity by allowing some level of suppression when a single mechanism is faulty. Additionally, it appears that CTLA-4 deficiency has its own internal safety measure, in that defective suppression due to CTLA-4 deletion can be partially overcome by increased expansion of Tfr cells.

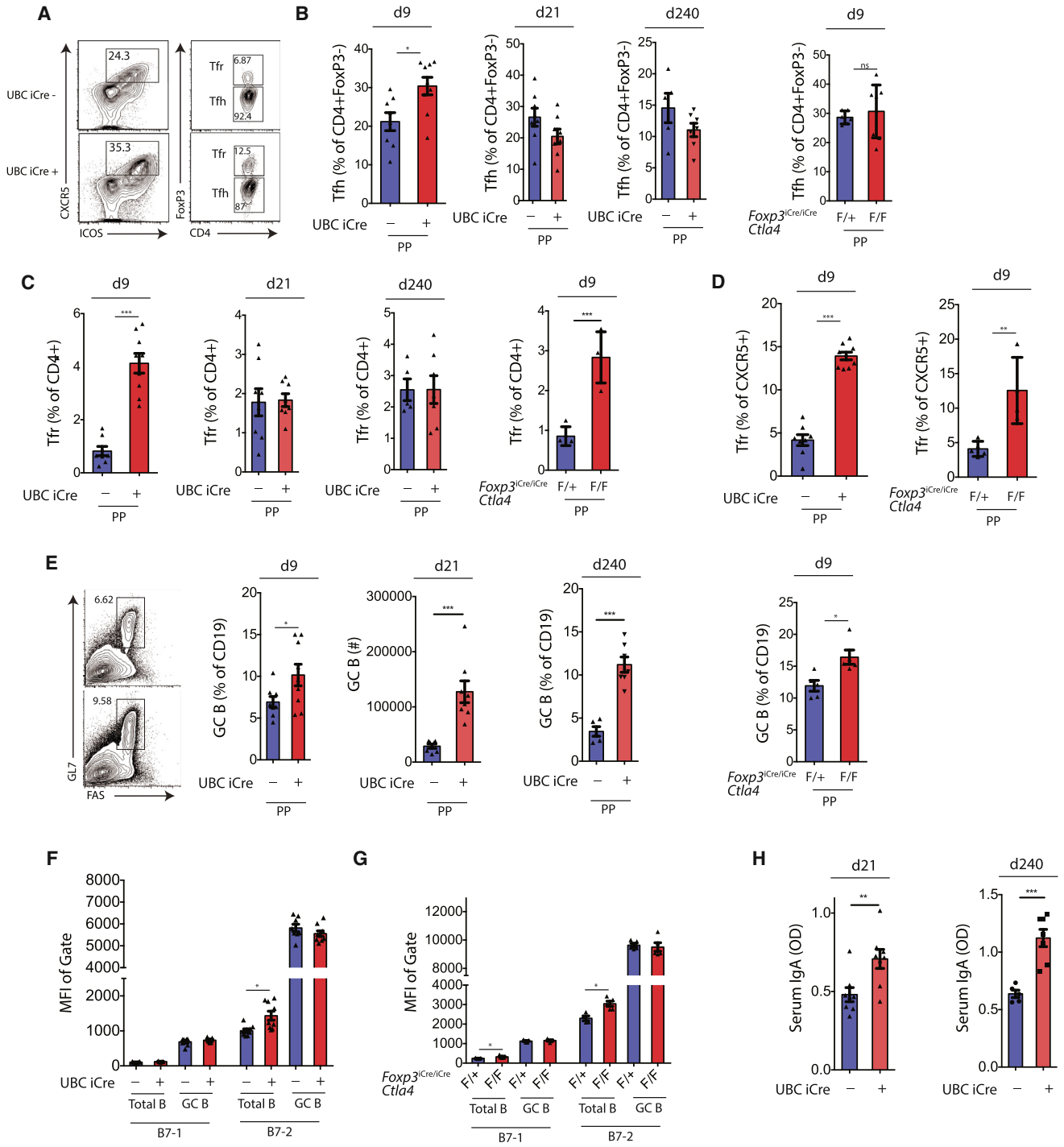

Figure 7. Deletion of CTLA-4 in Tfr Cells Results in Enhanced B Cell Responses in Peyer's Patches

(A) Representative gating of Tfh and Tfr cells from PP from UBC-iCre^{+} *Ctla4*$^{F/F}$ or UBC-iCre^{-} *Ctla4*$^{F/F}$ mice 9 days after the last dose of tamoxifen administration; tamoxifen was administered on days −2, −1, and 0.

(B) Quantification of Tfh cells (gated as in A) from UBC-iCre^{+}*Ctla4*$^{F/F}$ and UBC-iCre^{-}*Ctla4*$^{F/F}$ mice (left 3 panels) or *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/F}$ and *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/+}$ mice (far right panel) 9, 21 or 240 days after final tamoxifen treatment.

(C) Quantification of Tfr cells (gated as in A) from UBC-iCre^{+}*Ctla4*$^{F/F}$ and UBC-iCre^{-}*Ctla4*$^{F/F}$ mice (left three panels) or *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/F}$ and *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/+}$ mice (far right panel) reported as a percentage of CD4^{+} cells 9, 21, or 240 days after final tamoxifen treatment.

(D) Quantification of Tfr cells (gated as in A) from UBC-iCre^{+}*Ctla4*$^{F/F}$ and UBC-iCre^{-}*Ctla4*$^{F/F}$ mice (left) or *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/F}$ and *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/+}$ mice (right) reported as a percentage of total CD4^{+}CXCR5^{+} cells 9 days after final tamoxifen treatment.

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We did not observe altered B7-1 or B7-2 expression on GC B cells with CTLA-4-deleted Tfr cells in either our in vitro and in vivo suppression assays. We propose that Tfr cells in the GC suppress B cell responses through CTLA-4 using a mechanism that does not depend on B7-1 or B7-2 downregulation or transcytosis. It is likely that cell intrinsic signaling is responsible because we found changes in CTLA-4 deleted Tfr cells in vitro (such as increased ICOS expression). However, other mechanisms are also possible.

Deletion of CTLA-4 on Tfr cells enhanced Tfh and B cell responses in PP in addition to skin dLNs. Therefore, our data suggest that CTLA-4 modulation of Tfr and/or Treg cell suppressive function is not unique to skin dLNs, but instead is a general mechanism of suppression of B cell responses. Tfr suppression of IgA has been demonstrated in the gut; however, differences in IgA and GC B cells were found in the small intestine lamina propria, but not substantially in the PP (Kawamoto et al., 2014). We find that the deletion of CTLA-4 on Tfr and Treg cells in PP results in defective suppression, leading to heightened GC B responses and serum IgA levels. We hypothesize that this influences the gut microbiota similar to previous studies demonstrating altered IgA (Kawamoto et al., 2014; Kawamoto et al., 2012). Further experiments are necessary to investigate these possibilities.

Our work demonstrates that CTLA-4 controls B cell responses by regulating Tfh, Tfr, and Treg cells. Further work is needed to determine how modulating CTLA-4 in Tfh, Tfr, and/or Treg cells could be used to enhance vaccination responses and pathogen clearance.

EXPERIMENTAL PROCEDURES

Mice

For global inducible deletion of CTLA-4, UBC-ERT2-Cre (Jackson Laboratories) mice were crossed to *Ctla4* floxed mice to generate UBC-iCre *Ctla4*$^{F/F}$ mice (A.M.P. and A.H.S., unpublished data). These mice were also crossed to *Foxp3*$^{IRES\text{-}GFP}$ reporter mice to visualize FoxP3-expressing cells. UBC-iCre^{-} *Ctla4*$^{F/F}$ *Foxp3*$^{GFP/GFP}$ mice were used as controls for UBC-Cre^{+} *Ctla4*$^{F/F}$ *Foxp3*$^{GFP/GFP}$ experimental mice. For Treg cell-specific deletion of CTLA-4, *Foxp3*$^{ERT2\text{-}Cre\text{-}GFP}$ mice (Jackson Laboratories) were crossed to CTLA-4 floxed mice to generate *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/F}$ mice. *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/+}$ mice were used as controls for *Foxp3*$^{iCre/iCre}$ *Ctla4*$^{F/F}$ experimental mice to control for small alterations in Treg cell function due to the *Foxp3*^{iCre} allele. *Cd28*$^{-/-}$ and CD19-Cre mice were purchased from Jackson Laboratories. All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Health Guidelines.

Immunizations

Control and experimental mice were injected with 1 mg tamoxifen (Sigma) in sunflower oil intraperitoneally (i.p.) daily for 3 days. On the last day, mice were immunized subcutaneously (s.c.) on the flanks with 100 μg NP-OVA in a 1:1 emulsion in H37RA CFA. Mice were sacrificed and draining inguinal LNs were harvested. For unimmunized experiments, mice received five injections of tamoxifen daily and monitored for up to 240 days later. For in vitro assays of CTLA-4 effector function, UBC-Cre mice were immunized with NP-OVA s.c., 5 days later mice received one injection of tamoxifen i.p., and 3 days later organs were harvested for cell sorting. This strategy was used to maximize deletion of CTLA-4 at the start of in vitro assays, while simultaneously minimizing effects on differentiation, and was validated by comparing phenotypes of cells during sorting and by intracellular cytokine staining for CTLA-4.

Flow Cytometry

Cell suspensions were diluted in PBS with 1% FBS with 1 mM EDTA. Samples were preincubated with Fc block (BioLegend) and stained with the following directly labeled antibodies: CD4, CD19, ICOS, GL7, B7-1, B7-2, CD138, FAS, and PD-1. For CXCR5 staining, biotinylated CXCR5 (clone 2G8) was included in primary surface staining followed by streptavidin-conjugated Brilliant Violet 421 (BioLegend). For intracellular staining, the eBioscience FoxP3 intracellular staining kit was used. Samples were then incubated with anti-FoxP3, Ki67, Bcl6, CTLA-4, IRF4, IgG1, IL-17A, IFN-γ, IL-21, or IL-4. For cytokine analyses, samples were preincubated with 500 ng/ml Ionomycin and 250 ng/ml PMA in the presence of GolgiStop for 4 hr. All samples were analyzed on an LSRII or sorted on an Aria II with standard laser configurations.

In Vitro Stimulation Assays

For in vitro B cell stimulation assays with Tfh cells, 10–20 UBC-iCre^{-} or UBC-iCre^{+} mice were immunized with NP-OVA and 5 days later mice received one injection of tamoxifen. Three days later, the dLNs were harvested and CD4^{+} ICOS^{+}CXCR5^{+}FoxP3^{-}CD19^{-} Tfh cells were sorted. Tfh cells (3×10^4) were plated with 5×10^4 B cells (sorted as CD19^{+} cells from lymph nodes of NP-OVA immunized UBC-iCre^{-} mice) along with 2 μg/ml anti-CD3 (BioExcel), 5 μg/ml anti-IgM (Jackson Immunoresearch), and 0.25 nM 4-hydroxy-tamoxifen (4OHT; Sigma). Six days later, samples were analyzed.

In Vitro Suppression Assays

For in vitro B cell suppression assays with Tfr cells, UBC-iCre^{-} or UBC-iCre^{+} mice were immunized with NP-OVA and 5 days later mice received one injection of tamoxifen. Three days later, organs were harvested and 3×10^4 CD4^{+} ICOS^{+}CXCR5^{+}FoxP3^{-}CD19^{-} Tfh cells from iCre^{-} mice, 5×10^4 CD19^{+} B cells from iCre^{-}mice, and 1.5×10^4 CD4^{+}ICOS^{+}CXCR5^{+}FoxP3^{+}CD19^{-} Tfr cells from iCre^{-} or iCre^{+} mice were cultured in the presence of anti-CD3, anti-IgM, and 0.25 μM 4-hydroxy-tamoxifen (4OHT). Samples were analyzed 6 days later.

Adoptive Transfer Assays

For adoptive transfer studies of Tfr cells in which CTLA-4 was deleted, 20 FoxP3$^{iCre/iCre}$ CTLA-4$^{F/+}$ or FoxP3$^{iCre/iCre}$ CTLA-4$^{F/F}$ mice were immunized with NP-OVA s.c. Seven days later, mice received one injection of tamoxifen i.p., and 1 day later dLNs were harvested and 8×10^4 CD4^{+}ICOS^{+} CXCR5^{+}CD19^{-} (Tfh and Tfr cells) were adoptively transferred to CD28$^{-/-}$ mice, which were immunized the same day with NP-OVA and given tamoxifen i.p. Mice received an additional injection of tamoxifen 1 day later. Mice were sacrificed 9 days after NP-OVA immunization, and serum and dLNs were collected.

ELISA

ELISA assays to measure antibody and NP-specific IgG were performed as previously described (Sage et al., 2013).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.12.005.

(E) Increased germinal center B cells after Tfr deletion of CTLA-4 in PP. Identification of CD19^{+}FAS^{+}GL7^{+} B cells in UBC-iCre^{+}*Ctla4*$^{F/F}$ and UBC-iCre^{-}*Ctla4*$^{F/F}$ mice (representative gating, far left and quantification middle three panels) 9, 21, or 240 days or *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/F}$ and *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/+}$ mice (far right panel) 9 days after final tamoxifen treatment.
(F and G) B7-1 and B7-2 expression on CD19^{+} (Total B) or CD19^{+}GL7^{+}FAS^{+} (GC B) cells from PP as in (E) from UBC-iCre^{+}*Ctla4*$^{F/F}$ and UBC-iCre^{-}*Ctla4*$^{F/F}$ mice (F) or *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/F}$ and *Foxp3*$^{iCre/iCre}$*Ctla4*$^{F/+}$ mice (G) 9 days after final tamoxifen treatment.
(H) Serum IgA levels in UBC-iCre^{+}*Ctla4*$^{F/F}$ and UBC-iCre^{-}*Ctla4*$^{F/F}$ mice 21 or 240 days after last tamoxifen treatment. See also Figure S6.
All error bars contain SE.

AUTHOR CONTRIBUTIONS

P.T.S. conducted all experiments, A.M.P. and S.B.L. generated mice and provided technical help, and P.T.S. and A.H.S. designed, interpreted, and wrote the study.

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